(54) Title: HISTOLOGICAL RECONSTRUCTION AND AUTOMATED IMAGE ANALYSIS

(57) Abstract

A method for automated image analysis (Fig.
2, 42) of a biological specimen by histological recon-
struction, provided is an automated cell image method
for analyzing a biological specimen that has consec-
utively been stained by either an in situ hybridiza-
tion (ISH) method, or an immunohistochemistry (IHC)
method or a nucleic acid stain, and counterstained.
The method couples composite images in an automated
manner for processing and analysis to identify struc-
ture in tissue that cannot be captured in a single field
of view image (Fig. 2, 44) or a single staining tech-
nique, the disclosure provides a method for histolog-
ical reconstruction to analyze many fields of view on
potentially many slides (Fig. 2, 37) simultaneously.

HISTOLOGICAL RECONSTRUCTION AND AUTOMATED IMAGE ANALYSIS

TECHNICAL FIELD

The invention relates generally to light microscopy
and, more particularly, to automated techniques of
5   analyzing cytochemical and immunohistochemical staining.

BACKGROUND OF THE INVENTION

In current cellular imaging systems, the area of a
stained slide containing a stained cellular specimen is
10   scanned by automated microscopy systems. The entire
cellular specimen area of the slide is imaged with a
series of field of view images. For further analysis,
each field of view image must be separately analyzed to
locate complete candidate objects of interest within the
15   field of view image. This approach may be acceptable for
cellular objects or clusters that are not so large that
they extend beyond the field of view width or length of
an image. Often, for both automated and manual analysis,
only a single field of view is analyzed for morphological
20   characteristics, so that the context of the analysis is
limited to that field of view on a single slide.

SUMMARY OF THE INVENTION

A method for automated image analysis of a biological specimen is disclosed. A biological specimen is stained and counterstained for a specific marker or in the instance of immunohistochemistry or in situ hybridization, the marker is detectably labeled. Such labels include enzyme, radioisotopes, fluorescence or other labels well known in the art. The sample is then scanned at a plurality of positions by a photoimaging system to acquire an image. Adjacent positions are then used to reconstruct and provide a full picture image of the whole sample. A reconstructed whole sample image may then be further processed to identify coordinates that may have an object or area of interest. These coordinates are automatically selected for a candidate object of interest. In a preferred embodiment, a low magnification image of the candidate objects of interest is automatically obtained. Preferably the image is a color digital image. The candidate object of interest pixels in each sample are automatically morphologically processed to identify artifact pixels and the remaining candidate object of interest pixels in the sample not identified as artifact pixels. The apparatus obtaining the low magnification image is adjusted to a higher

magnification, to acquire a higher magnification image of the sample, at the location coordinates corresponding to the low magnification image, for each candidate object or area of interest. Pixels of the higher magnification

5   image in the first color space are automatically transformed to a second color space to differentiate higher magnification candidate objects of interest pixels from background pixels and identify, at higher magnification, objects of interest from the candidate

10   object of interest pixels in the second color space. Thus, the pathologist or technician can identify whether the candidate object of interest has been specifically stained for the marker of interest, or counterstained, or both specifically stained and counterstained.

15   An automated cellular image method for analyzing a biological specimen, that has consecutively been stained with hematoxylin and eosin (H/E) on one tissue section and by one or several immunohistochemistry (IHC) and/or in situ hybridization (ISH) methods on parallel tissue

20   sections, is also disclosed as a particular embodiment. To identify a structure in tissue that cannot be captured in a single field of view image or a single staining technique, the invention provides a method for histological reconstruction to analyze many fields of

view on potentially many slides simultaneously. The
method couples composite images in an automated manner
for processing and analysis. A slide on which is mounted
a biological specimen stained to identify structure of

5   interest is supported on a motorized stage. An image of
the biological specimen is generated, digitized, and
stored. As the viewing field of the objective lens is
smaller than the entire biological specimen, a
histological reconstruction is made. These stored images

10 of the entire tissue section may then be placed together
in an order such that the H/E stained slide is paired
with the immunohistochemistry slide so that analysis of
the images may be performed simultaneously by the
pathologist. The images may be superimposed or viewed as

15 adjacent images.

In one embodiment, the invention provides a method
for automated image analysis of a biological specimen by
providing a biological sample to be analyzed,
automatically scanning the sample at a plurality of

20 coordinates, automatically obtaining an image at each of
the coordinates, reconstructing an image of the sample
from each individual image to create a reconstructed
image and processing the reconstructed image to identify
a candidate object or area of interest.

In another embodiment, the invention provides a
method for histological reconstruction. In this method a
sample of a biological specimen is divided into a number
of subsamples. Each subsample is processed with a stain,
5 counterstain, immunohistochemical technique, in situ
hybridization technique, or a combination thereof. Each
sample is then scanned and an image is obtained from each
of the samples. The images are then reconstructed such
that a first image is paired with a consecutive-
10 corresponding sample image for identification of objects
or areas of interest.

In yet another embodiment, the invention provides a
computer program residing on a computer-readable medium,
for automated image analysis of a biological specimen.
15 The computer program comprises instructions for causing a
computer to process a sample by scanning the sample at a
plurality of coordinates, obtaining an image at each of
the coordinates, reconstructing the sample from the
individual images to create a reconstructed sample,
20 identifying a coordinate of a candidate object or area of
interest in the reconstructed image and acquiring an
image at the coordinate obtained from the reconstructed
image.

6

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of an apparatus for automated cell image analysis.

FIG. 2 is a block diagram of the apparatus shown in
5   FIG. 1.

FIG. 3 is a plan view of the apparatus of FIG. 1 having the housing removed.

FIG. 4 is a side view of a microscope subsystem of the apparatus of FIG. 1.

10   FIG. 5 shows a slide carrier. FIG. 5a is a top view of a slide carrier for use levity the apparatus of FIG. 1. FIG. 5b is a bottom view of the slide carrier of FIG. 5a.

FIG. 6 shows views of an automated slide handling
15   subsystem. FIG. 6a is a top view of an automated slide handling subsystem of the apparatus of FIG. 1. FIG. 6b is a partial cross-sectional view of the automated slide handling subsystem of FIG. 6a taken on line A-A.

FIG. 7a-7d illustrate the output operation of the
20   automated slide handling subsystem.

FIG. 8 is a flow diagram of the procedure for automatically determining a scan area.

FIG. 9 is a block diagram of the microscope controller of FIG. 2

FIG. 10 shows a method of histological reconstruction.

DETAILED DESCRIPTION

Overview

5   An automated microscope for analyzing cellular specimens is shown in FIG's. 1 and 3 and in block diagram in FIG. 2. A motorized stage 38 may be used to support a slide 70 (FIG. 5). On the slide is mounted a biological specimen that is typically stained to identify a

10   structure of interest for analysis. A biological specimen is defined as a cellular or acellular sample of biological origin, which has been mounted on a microscope slide. The biological specimen can be, for example, a tissue section, a biological fluid specimen, for example,

15   a blood fraction cytospun on a microscope slide or a cell suspension directly seeded on a slide.

At least one optical sensing array, such as an objective lens, 44a is located above the stage and a light source 48 is located beneath the stage. Light from

20   the source illuminates the stage and slide so an image of the biological specimen is generated by the optical sensing array, for example an objective lens. This image is stored in memory. Preferably the image is a digitized or digital image. As the viewing field of the optical

array is smaller than the entire biological specimen, the stage is moved in one planar direction by a distance that corresponds to the length of the field of view in that direction.   The image generated at that position may then

5   be captured and stored.   The acquired image may be flipped along its centerline due to the optical flipping of the original image.   Movement of the stage and capture of the resulting image continues in the same direction until the end of the specimen area of the slide is

10   reached.   At that time, the stage is moved in the other planar direction by a distance that corresponds to the length of the field of view in that direction and another image is generated and stored.   The slide is traversed or scanned in this manner until the entire specimen area of

15   the slide has been viewed through the objective lens. These stored images may then be placed together in the order in which they were collected to generate a composite or reconstructed image of the cellular specimen.   This composite image may then be analyzed to

20   detect a structure that extends across more than one image field or more than one slide for further analysis. Such analysis may result in the identification of a candidate object or area of interest in both the field of view as well as objects that overlap two or more fields

9

of view. In such instances, the system will

automatically determine the coordinates for these

candidate objects and may obtain additional images at

various magnifications.

5   In existing cellular imaging systems, the area of the

slide is scanned by automated microscopy systems to image

the entire specimen area of the slide with a series of

field of view images in the manner described above.

However, these systems require that each field of view

10   image is separately analyzed to locate complete candidate

objects of interest within the field of view image for

further analysis. This approach is acceptable for

cellular objects that are not so large that they extend

beyond the field of view width or length of an image. To

15   identify structure in tissue that cannot be captured in a

single field of view image, the present system uses an

analysis method to identify field of view images that

appear to contain part of a tissue structure to be

analyzed. Field of view images so identified that are

20   adjacent to one another are then identified as containing

the tissue structure that the stain, antibody, or probe

was intended to identify. This portion of the composite

image may then be viewed under a higher magnification

power for additional detail. The automatic building of a

composite specimen image from the field of view images
and the processing of the composite image or its
constituent images to identify tissue structure that
extends beyond one field of view is unknown.

5   In addition, a problem with existing automated
systems is the continued need for operator input to
initially locate cell objects for analysis. Such
continued dependence on manual input can lead to errors
including objects of interest being missed. These errors
10   can be critical especially in assays for so-called rare
events, e.g., finding one stained cell in a cell
population of one million normal cells. Additionally,
manual methods can be extremely time consuming and can
require a high degree of training to properly identify or
15   quantify cells. The associated manual labor leads to a
high cost for these procedures in addition to the
potential errors that can arise from long, tedious manual
examinations. A need exists, therefore, for an improved
automated cell image analysis system which can quickly
20   and accurately scan large amounts of biological material
on a slide.

Unless otherwise defined, all technical and
scientific terms used herein have the same meaning as
commonly understood by one of ordinary skill in the art

to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described

5   below.

Nuclear Stains, Intercalating Dyes and Counterstains

The term "nuclear stain" refers to a cytochemical stain that preferentially stains the nuclei of eukaryotic cells. Many nuclear stains are intercalating dyes. The

10   term "intercalating dye" refers to a chemical compound that can insert itself in between adjacent nucleotides of a nucleic acid to provide a detectable color.

Many nuclear stains are known in the art, with one of the most commonly used being hematoxylin. Hematoxylin is

15   often used in combination with various metallic salts (mordants). Hematoxylin stains are used for different staining purposes, and have a variety of colors, depending on the metal used. Aluminum lakes are purple to blue, depending on pH. Iron lakes are blue-black.

20   Chromium lakes are blue-black. Copper lakes are blue-green to purple. Nickel lakes are various shades of violet. Tin lakes are red. Lead lakes are dark brown. Osmium lakes are greenish brown. Other nuclear stains

include Giemsa stain, methyl green (which binds to AT-
rich DNA regions), and Nuclear Fast-Red.

Fluorescent stains include Hoechst 33342; Hoechst
33258 (Calbiochem), a bisbenzimide DNA intercalator that
5 excites in the near UV (350 nm) and emits in the blue
region (450 nm); thiazole orange, a fluorogenic stain for
DNA that excites in the blue region (515 nm) and emits in
the green region (530 nm) of the visible spectrum; DAPI;
ethidium bromide; propidium iodide; TOTO; YOYO_1; and
10 SYTOX Blue or Green stains are also encompassed by the
current invention. Several dyes either bind GC-rich or
AT-rich chromosomal regions preferentially or show
differences in fluorescence intensity upon binding those
regions, yielding fluorescent banding patterns. For
15 example, 7-Aminoactinomycin D binds selectively to GC-
rich DNA regions and. 9-Amino-6-chloro-2-methoxyacridine
fluoresces with greatest intensity in AT-rich DNA
regions. Acridine homodimer fluoresces preferentially
when bound to AT-rich DNA regions.

20   The term "counterstain," when used in combination
with nuclear stains, refers to cytochemical stains that
bind to a region of a eukaryotic cell other than the
nucleus. Many counterstains are known in the art. One of
the most common is eosin, which stains eukaryotic cell

cytoplasm to varying shades of pink. Other counterstains
are specific for a particular organelle or a protein in a
cell. For example, the Kleihauer-Betke cytochemical stain
is specific for hemoglobin F, a hemoglobin type

5   preferentially expressed in fetal cells and therefore can
be defined as a specific marker of fetal red blood cells.
The term "coordinate" or "address" is used to mean a
particular location on a slide or sample. The coordinate
or address can be identified by any number of means

10   including, for example, X-Y coordinates, r-P coordinates,
and others recognized by those skilled in the art.

     In one embodiment, an automated cellular imaging
method is used to identify fetal nucleated red blood
cells in a maternal blood specimen. Fetal cells are first

15   identified following the Kleihauer-Betke cytochemical
stain for hemoglobin F. Fetal cells are identified by the
automated cellular imaging system as objects on the basis
of their bright red color (indicative of Hemoglobin F) as
compared to maternal red blood cells. To assure that

20   appropriate objects are identified, size and shape
morphological "filters" are used to exclude very small
and very large objects.

     Cells are counterstained with an additional
cytochemical stain for nucleic acids, resulting in a blue

color for nucleated red blood cells (generally, fetal red
blood cells). An automated image analysis system
identifies blue objects of the appropriate size and shape
for an erythrocyte nucleus among the bright red objects,

5   allowing the imaging system to identify and enumerate
nucleated fetal red cells. Such cells can be enumerated,
allowing for a screen for Down's syndrome in the fetus,
wherein the frequency of such cells is typically higher
in Down's syndrome pregnancies compared with normal

10   pregnancies.

In a preferred embodiment, the slides are stained
with hematoxylin/eosin (H/E) and one or several parallel
slides containing adjacent sections are stained for one
or several specific markers.

15   The results of the H/E staining provide cells with
nuclei stained blue-black, cytoplasm stained varying
shades of pink; muscle fibers stained deep pinky red;
fibrin stained deep pink; and red blood cells stained
orange-red.

20   For example, hematoxylin/eosin (H/E) slides are
prepared with a standard H/E protocol. Standard
solutions include the following: (1) Gills hematoxylin
(hematoxylin 6.0 g; aluminum sulphate 4.2 g; citric acid
1.4 g; sodium iodate 0.6 g; ethylene glycol 269 ml;

distilled water 680 ml); (2) eosin (eosin yellowish 1.0

g; distilled water 100 ml); (3) lithium carbonate 1%

(lithium carbonate 1 g; distilled water 100 g); (4) acid

alcohol 1% 70% (alcohol 99 ml conc.; hydrochloric acid 1

5   ml); and (5) Scott's tap water. In a beaker containing 1

L distilled water, add 20 g sodium bicarbonate and 3.5 g

magnesium sulphate. Add a magnetic stirrer and mix

thoroughly to dissolve the salts. Using a filter funnel,

pour the solution into a labeled bottle.

10   The staining procedure is as follows: (1) Bring the

tissue or cell sections to water; (2) place sections in

hematoxylin for 5 minutes (min); (3) wash in tap water;

(4) 'blue' the sections in lithium carbonate or Scott's

tap water; (5) wash in tap water; (6) place sections in

15   1% acid alcohol for a few seconds; (7) wash in tap water;

(8)place sections in eosin for 5 min; (9) wash in tap

water; and (10) dehydrate with graded alcohol solution.

Mount sections.

          A specific marker is a molecule or a group of

20   molecules, which is present in only a subset of the

components of a biological specimen and therefore

identifying specifically the components having the

marker. Specific markers are frequently defined as

antigens recognized by specific antibodies (monoclonals or polyclonals) and can be detected by immunohistochemistry.

Another group of specific markers is defined by the capacity of these markers to hybridize, specifically, a nucleic acid probe. These markers can usually be detected by in situ hybridization.

A third group of specific markers can be defined by their enzymatic activity and can be detected by histochemistry.

A fourth group of specific markers can be stained directly, histochemically, using a specific dye.

A fifth group of specific markers can be defined as being receptors binding specifically to one or several ligands. A specific ligand is itself used for the detection of the receptor-ligand complex, using a detection method involving either histochemistry, or immunohistochemistry or in situ hybridization.

Immunohistochemical and In Situ Hybridization Techniques

Immunohistochemical techniques as used herein encompasses the use of reagents detecting cell specific markers, such reagents include, for example, as antibodies and nucleic acids probes. Antibodies,

including monoclonal antibodies, polyclonal antibodies
and fragments thereof, are often used to identify
proteins or polypeptides of interest in a sample. A
number of techniques are utilized to label objects of
5   interest according to immunohistochemical techniques.
Such techniques are discussed in Current Protocols in
Molecular Biology, Unit 14 et seq., eds. Ausubel, et al.,
John Wiley & Sons, 1995, the disclosure of which is
incorporated herein by reference. For example, the
10 following procedure is an example of immunohistochemical
staining using an antibody recognizing, specifically, the
HER2 protein. HER2 overexpression has been described as
a specific marker in a high percentage of breast cancer
carcinomas. This protocol is intended for staining a
15 paraffin embedded tissue section prepared according to a
conventional procedure.

       The section is deparaffinized using two baths of
xylene and rehydrated through graded alcohols baths and
finally in deionized water. The section is then
20 incubated with an Antigen Retrieval Buffer, containing
Citrate, for 40 minutes at 95°C. The slide is then
cooled down at room temperature for 20 minutes in the
same buffer. The slide is then rinsed in deionized
water.

The area surrounding the tissue section is carefully
dried and a hydrophobic delimiting pen is used to draw a
line around the specimen, on the glass slide. A
peroxidase blocking solution is added on the section and
5   incubated 5 minutes at room temperature. After being
washed twice with the Wash Buffer (a balanced salt
solution), the tissue section is incubated 30 minutes at
room temperature, with the primary antibody recognizing
the HER2 protein.

10   After 3 washes with the Wash Buffer, the tissue
section is incubated with the peroxidase conjugated
secondary antibody. The secondary antibody will
recognize specifically the primary antibody. The slide
is then washed 3 times with the Wash Buffer. Then the
15   tissue section is incubated in presence of DAB and
hydrogen peroxide for 10 minutes, before being washed
with water.

The tissue section is counterstained in Hematoxylin
for 2 minutes and rinsed again with water. The slide is
20   mounted with a cover-slip using an aqueous mounting
medium.

Immunohistochemical localization of cellular
molecules uses the ability of antibodies to bind specific
antigens, for example proteins of interest such as onco-

proteins and enzymes, with high affinity. These
antibodies can be used to localize antigens to
subcellular compartments or individual cells within a
tissue.

5   In situ hybridization techniques include the use of
specifically labeled nucleic acid probes, which bind to
cellular RNA or DNA in individual cells or tissue
section. Suitable nucleic acid probes may be prepared
using standard molecular biology techniques including
10   subcloning, plasmid preparation, and radiolabeling or
non-radioactive labeling of the nucleic acid probe.

In situ hybridization is often performed on either
paraffin or frozen sections. Such techniques often
include fine sectioning of tissues to provide samples
15   that are only a single to a few cell layers thick. For
example paraffin blocks containing a tissue sample are
cut into thin, approximately 8 um tissue sections, which
are subsequently mounted on subbed slides to be further
processed for in situ hybridization. Alternatively,
20   methacrylate may be used for sectioning. Cryosectioning
techniques are particularly suitable for
immunohistochemistry and enzyme histochemistry.

Immunofluorescent labeling of a tissue section often
use a sandwich assay or a primary antibody and secondary

antibody-fluorochrome conjugate. Slides containing a
tissue section of interest are washed in phosphate
buffered saline and then exposed to a primary antibody
which will bind to the protein object of interest.

5   Subsequently the slides are washed and exposed to the
secondary antibody which binds to the first or primary
antibody. The slide is washed and then developed.
Numerous other techniques well known in the art of
immunohistochemical staining and in situ hybridization

10   are easily adaptable for use in immunohistochemical
reconstruction as disclosed herein.

Thus, a combination of techniques using both chemical
staining and/or immunohistochemical and/or in situ
hybridization may be used in the present methods. For

15   example, numerous subsamples may be prepared from a
single tissue specimen. A first subsample may be
chemically stained as discussed above, and a subsequent
subsample may be subjected to immunohistochemical and in
situ hybridization techniques. Each subsamples is

20   scanned and processed as discussed below. Reconstructed
images may then be overlapped or processed to further
identify objects of interest.

Histological Reconstruction

Histological reconstruction is a process whereby an image of a whole specimen is constructed from analyzed pieces of the specimen, particularly when the biological

5   specimen has been mounted on a slide. This image is created by piecing together more than one field of view at any particular magnification.

With reference to Fig. 10, an image 302, representing an objective's field of view is acquired at a first

10   particular coordinate on the slide sample 301. The slide is automatically repositioned on the X-Y stage to obtain a new or second field of view corresponding to a second particular coordinate 303. This new field of view is preferably immediately adjacent to the first field of

15   view, however, so long as the coordinates, thus the address/identity, of each field of view are retained in the imaging system, histological reconstruction may be performed. This process is repeated until images for the whole of the specimen have been acquired.

20   Based upon each image's X and Y coordinate, the specimen is digitally reconstructed. As part of the reconstruction, the image may be flipped to correct for the optical flipping of the original image.

The process of forming a histological reconstructed image involves having the apparatus scan a microscope slide of interest, and form the image that constitutes a reconstruction of the images taken during the scan. The

5   image that is formed can be a full-color reconstruction of the entire scan area, or a fraction of the whole scan area, for example, reconstruction of the entire scan area that identifies objects or areas of interests. The reconstructed digital image can then be used for further

10 processing or analysis to identify previously undetected objects or areas of interest. For example, objects or areas of interest overlapping one or more fields of view or slides may thus be identified in the reconstructed digital image.

15 With reference to FIGURES 1 and 2, the apparatus 10 also referred to as the system, comprises a microscope 32 with a motorized X, Y and Z stage 38, a camera 42, a computer 22 adapted to receive and process video images, and a set of software programs to control the apparatus

20 and to execute the method. A measurement of the optical properties of the sample features is used to form an image of the scannable area of the slide, to find sub-regions of interest, and to analyze the properties of these regions. The image processing method that

evaluates the sample to find regions of interest uses a

measure of the hue, saturation or intensity and

luminosity of a 24-bit color image to produce a white on

black target image of interest. This image is processed

5   by separately converting the full color image to

components of hue, saturation or intensity and

luminosity, thresholding the components, and performing a

logical "AND" between the two images, then thresholding

the resulting image such that any pixel value above zero

10   becomes 255. The processing and image acquisition will

be further understood with reference to the apparatus

described below.

       Automated System

15   The invention provides a method for automated

analysis of a biological specimen, which eliminates the

need for operator input to locate biological objects or

areas of interest for analysis.

        With reference to FIGURE 1, a slide prepared with a

20   biological specimen and reagent is placed in a slide

carrier 60 (FIG. 5) which preferably holds four slides.

The slide carriers are loaded into an input hopper 16 of

the automated system 10. The operator then enters data

identifying the instrument protocol which contains

information on the size, shape and location of a scan

area on each slide, or, preferably, the system

automatically locates a scan area for each slide during

slide processing. The operator then activates the system

5   10 for slide processing. At system activation, a slide

carrier 60 is positioned on an X-Y stage 38 of an optical

system, such as microscope subsystem 32. Any bar codes

used to identify slides are read and stored for each

slide in the carrier. The entire slide is rapidly

10 scanned at a low magnification, typically 10x. At each

location of the scan, a low magnification image is

acquired and processed to detect candidate objects or

areas of interest. Preferably, color, size and shape are

used to identify objects or areas of interest. The

15 location of each candidate object or area of interest may

be stored by reference to its coordinates or address.

Each field of view may also be stored as part of a larger

composite image (described in detail elsewhere).

    At the completion of the low level scan for each

20 slide in the carrier on the stage, the optical system may

be adjusted to a higher magnification such as 40x or 60x,

for additional specimen processing and image acquisition,

and the X-Y stage is positioned to the stored locations

for the candidate objects or areas of interest on each

slide in the carrier. A higher magnification image is
acquired for each candidate object or area of interest
and a series of image processing steps are performed to
confirm the analysis, which was performed at low

5 magnification. A higher magnification image is stored
for each continued object or area of interest. These
images are then available for retrieval by a pathologist
or cytotechnologist to review for final diagnostic
evaluation. Having stored the location of each object or

10 area of interest, a mosaic comprising the candidate
objects or areas of interest for a slide may be generated
and stored. The pathologist or cytotechnologist may view
the mosaic or may also directly view the slide at the
location of an object or area of interest in the mosaic

15 for further evaluation. The mosaic may be stored on
magnetic or optical media for future reference or may be
transmitted to a remote site for review or storage. The
entire process involved in examining a single slide takes
on the order of 4-100 min depending on scan area size and

20 the number of detected candidate objects of interest.

       The invention has utility in prenatal diagnosis of
fetal cells, rare event detection, rapid cell counting,
tissue evaluation, and other diagnoses.

The processing of images acquired in the automated
scanning preferably includes the steps of transforming
the image to a different color space, here preferably by
hue, saturation and intensity. The pixels of the filtered
5   image are dynamically thresholded to suppress background
material; performing a morphological function to remove
artifacts from the thresholded image; analyzing the
thresholded image to determine the presence of one or
more regions of connected pixels having the same color;
10   and categorizing every region having a size greater than
a minimum size as a candidate object or area of interest.

According to another aspect, the scan area is
automatically determined by scanning the slide; acquiring
an image at each slide position; analyzing texture or
15   color information for each image to detect the edges of
the specimen and storing the locations corresponding to
the detected edges to define the scan area.

According to yet another aspect, automated focusing
of the optical system is achieved by initially
20   determining a focal surface from an array of points or
locations in the scan area. The derived focal surface
enables subsequent rapid automatic focusing in the low
power scanning operation. In one embodiment, the focal
plane is determined by determining proper focal positions

across an array of locations and performing a least
squares fit of the array of focal positions to yield a
focal plane across the array. Preferably, a focal
position at each location is determined by incrementing
5   the position of a Z stage for a fixed number of coarse
and fine iterations. At each iteration, an image is
acquired and a pixel variance, morphological gradient or
other optical parameter about a pixel mean for the
acquired image is calculated to form a set of evaluation
10   data. The peak value of the least squares fit curve is
selected as an estimate of the best focal position.

In another aspect, a focal position method for a
higher magnification locates a region of interest
centered about a candidate object of interest within a
15   slide which was located during an analysis of the low
magnification images. The region of interest is
preferably n columns wide, where n is a power of 2. The
pixels of this region are then processed using a Fast
Fourier Transform to generate a spectra of component
20   frequencies and corresponding complex magnitude for each
frequency component. Preferably, the complex magnitude
of the frequency components which range from 25% to 75%
of the maximum frequency component are squared and summed
to obtain the total power for the region of interest.

This process is repeated for other Z positions and the Z

position corresponding to the maximum total power for the

region of interest is selected as the best focal

position. This focal method can be used with many stains

5   and types of cellular specimens.

According to still another aspect, a method for

automated slide handling is provided. A slide is mounted

onto a slide carrier 60 (FIG. 5) with a number of other

slides side-by-side. The slide carrier 60 is positioned

10   in an input feeder 16 with other slide carriers to

facilitate automatic analysis of a batch of slides. The

slide carrier is loaded onto the X-Y stage 38 of the

optical system 32 for the analysis of the slides thereon.

Subsequently, the first slide carrier is unloaded into an

15   output feeder 18 after automatic image analysis and the

next carrier is automatically loaded.

Referring to the FIGURES, an apparatus for automated

cell image analysis of biological specimens is generally

indicated by reference numeral 10 as shown in perspective

20   view in FIG. 1 and in block diagram form in FIG. 2. The

apparatus 10 comprises a microscope subsystem 32 housed

in a housing 12. The housing 12 includes a slide carrier

input hopper 16 and a slide carrier output hopper 18. A

door 14 in the housing 12 secures the microscope

subsystem from the external environment. A computer
subsystem comprises a computer 22 having two system
processors 23, an image processor 25 and a communications
modem 29. The computer subsystem further includes a

5   computer monitor 26 and an image monitor 27 and other
external peripherals including storage device 21,
pointing device 30, keyboard 28 and color printer 35. An
external power supply 24 is also shown for powering the
system. Viewing oculars 20 of the microscope subsystem

10 project from the housing 12 for operator viewing. The
apparatus 10 further includes a 3-chip CCD camera 42 for
acquiring images through the microscope subsystem 32.
The computer directly controls a number of microscope
subsystem functions described further in detail.

15 An automatic slide feed mechanism 37 in conjunction with
X-Y stage 38 provide automatic slide handling in the
apparatus 10. An illumination light source 48 projects
light onto the X-Y stage 38 which is subsequently imaged
through the microscope subsystem 32 and acquired through

20 the 3-chip CCD camera 42 for processing by the image
processor 25. A Z stage or focus stage 46 under control
of the microscope controller 31 provides displacement of
the microscope subsystem in the Z plane for focusing.

The microscope subsystem 32 further includes a motorized
objective turret 44 for selection of objectives.

The apparatus 10 is for the unattended automatic
scanning of prepared microscope slides for the detection
5 and counting of candidate objects or areas of interest,
such as stained cells. In one embodiment the invention
may be utilized for tissue analysis. In another
embodiment, rare event detection in which there may be
only one candidate object of interest per several hundred
10 thousand normal cells, e.g., one to five candidate
objects of interest per 2 square centimeter area of the
slide. The apparatus 10 automatically locates and counts
candidate objects or areas of interest and estimates
normal cells present in a cellular specimen on the basis,
15 for example, of color, size and shape characteristics. A
biological specimen may be prepared with a reagent to
obtain a colored insoluble precipitate. The apparatus, in
one embodiment, is used to detect this precipitate as a
candidate object or area of interest.

20 During operation of the apparatus 10, a pathologist
or laboratory technician mounts prepared slides onto
slide carriers. A slide carrier 60 is illustrated in
FIG. 5 and is described further below. Each slide
carrier holds up to 4 slides. Up to 25 slide carriers

31

are then loaded into input hopper 16. The operator can
specify the size, shape and location of the area to be
scanned or alternatively, the system can automatically
locate this area. The operator then commands the system
to begin automated scanning of the slides through a
graphical user interface. Unattended scanning begins
with the automatic loading of the first carrier and slide
onto the motorized X-Y stage 38. A bar code label
affixed to the slide is read by a bar code reader 33
during this loading operation. Each slide is then
scanned at a user selected low microscope magnification,
for example, 10x, to build a histological reconstruction
or identify candidate objects based on their color, size
and shape characteristics. The X-Y locations of
candidate objects or areas of interest are stored until
scanning is completed.

        After the low magnification scanning is completed,
the apparatus may automatically return to each candidate
object or area of interest, if necessary, reimaging and
refocusing at a higher magnification such as 40x and
performs further analysis to confirm the biological
candidate. The apparatus stores an image of the object
or area of interest for later review by a pathologist.
All results and images can be stored to a storage device

21 such as a removable hard drive or optical disc or DAT

tape or transmitted to a remote site for review or

storage. The stored images for each slide can be viewed

in a mosaic of images for further review. In addition,

5   the pathologist or operator can also directly view a

detected object or area of interest through the

microscope using the included oculars 20 or on image

monitor 27.

The two system processors 102 further control an

10   illumination controller 106 for control of substage

illumination 48. The light output from, for example, a

halogen light bulb, which supplies illumination for the

system, can vary over time due to bulb aging, changes in

optical alignment, and other factors. In addition,

15   slides which have been "over stained" can reduce the

camera exposure to an unacceptable level. To compensate

for these effects, the illumination controller 106 is

included. This controller is used in conjunction with

light control software to compensate for the variations

20   in light level. The light control software samples the

output from the camera at intervals (such as between

loading of slide carriers), and commands the controller

to adjust the light level to the desired levels. In this

way, light control is automatic and transparent to the
user and adds no additional time to system operation.

The system processors 23 are preferably comprised of
dual parallel Intel Pentium Pro 400 MHZ devices. The
5   image processor 25 is preferably a Matrox Genesis board.
The computer, in a preferred embodiment, operates under
Windows NT. It will be recognized that any number of
processors and operating systems can be used in the
methods and in conjunction with the present invention.

10   Referring now to FIG. 3 and 4, further detail of the
apparatus 10 is shown. FIG. 3 shows a plan view of the
apparatus 10 with the housing 12 removed. A portion of
the automatic slide feed mechanism 37 is shown to the
left of the microscope subsystem 32 and includes slide
15   carrier unloading assembly 34 and unloading platform 36
which in conjunction with slide carrier unloading hopper
18 function to receive slide carriers which have been
analyzed.

Vibration isolation mounts 40, shown in further
20   detail in FIG. 4, are provided to isolate the microscope
subsystem 32 from mechanical shock and vibration that can
occur in a typical laboratory environment. In addition
to external sources of vibration, the high speed
operation of the X-Y stage 38 can induce vibration into

the microscope subsystem 32. Such sources of vibration
can be isolated from the electro-optical subsystems to
avoid any undesirable effects on image quality. The
isolation mounts 40 comprise a spring 40a and piston 40b
5   submerged in a high viscosity silicon gel which is
enclosed in an elastomer membrane bonded to a casing to
achieve damping factors on the order of 17% to 20%.

The automated slide handling subsystem operates on a
single slide carrier at a time. A slide carrier 60 is
10   shown in FIG. 5a & 5b, which provide a top view and a
bottom view respectively. The slide carrier 60 includes
up to four slides 70 mounted with adhesive tape 62. The
carrier 60 includes ears 64 for hanging the carrier in
the output hopper 18. An undercut 66 and pitch rack 68
15   are formed at the top edge of the slide carrier 60 for
mechanical handling of the slide carrier. A keyway
cutout 65 is formed in one side of the carrier 60 to
facilitate carrier alignment. A prepared slide 72
mounted on the slide carrier 60 includes a sample area
20   72a and a bar code label area 72b.

FIG. 6a provides a top view of the slide handling
subsystem which comprises a slide input module 15, a
slide output module 17 and X-Y stage drive belt 50. FIG.

6b provides a partial cross-sectional view taken along
line A-A of FIG. 6a.

   The slide input module 15 includes a slide carrier
input hopper 16, loading platform 52 and slide carrier
5   loading subassembly 54. The input hopper 16 receives a
series of slide carriers 60 (FIG. 5a and 5b) in a stack
on loading platform 52. A guide key 57 protrudes from a
side of the input hopper 16 to which the keyway cutout 65
(FIG. 5a) of the carrier is fit to achieve proper
10 alignment.

   The input module 15 further includes a revolving
indexing cam 56 and a switch 90 mounted in the loading
platform 52, the operation of which is described further
below. The carrier subassembly 54 comprises an infeed
15 drive belt 59 driven by a motor 86. The infeed drive
belt 59 includes a pusher tab 58 for pushing the slide
carrier horizontally toward the X-Y stage 38 when the
belt is driven. A homing switch 95 senses the pusher tab
58 during a revolution of the belt 59.

20   Referring specifically to FIG. 6a, the X-Y stage 38
is shown with x position and y position motors 96 and 97
respectively which are controlled by the microscope
controller 31 (FIG. 9) and are not considered part of the
slide handling subsystem. The X-Y stage 38 further

includes an aperture 55 for allowing illumination to
reach the slide carrier. A switch 91 is mounted adjacent
the aperture 55 for sensing contact with the carrier and
thereupon activating a motor 87 to drive stage drive belt

5   50 (FIG. 6b). The drive belt 50 is a double-sided timing
belt having teeth for engaging pitch rack 68 of the
carrier 60 (FIG. 5b).

The slide output module 17 includes slide carrier
output hopper 18, unloading platform 6, and slide carrier

10   unloading subassembly 34. The unloading subassembly 34
is a motor 89 for rotating the unloading platform 36
about shaft 98 during an unloading operation described
further below. An outfeed gear 93 driven by motor 88
rotatably engages the pitch rack 68 of the carrier 60

15   (FIG. 5b) to transport the carrier to a rest position
against switch 92. A spring loaded hold-down mechanism
holds the carrier in place on the unloading platform 36.

The slide handling operation is now described.
Referring to FIG. 7, a series of slide carriers 60 are

20   shown stacked in input hopper 16 with the top edges 60a
aligned. As the slide handling operation begins, the
indexing cam 56 driven by motor 85 advances one
revolution to allow only one slide carrier to drop to the
bottom of the hopper 16 and onto the loading platform 52.

FIG. 7a-7d show the cam action in more detail. The indexing cam 56 includes a hub 56a to which are mounted upper and lower leaves 56b and 56c respectively. The leaves 56b & 56c are semicircular projections oppositely

5   positioned and spaced apart vertically. In a first position shown in FIG. 8a, the upper leaf 56b supports the bottom carrier at the undercut portion 66. At a position of the indexing cam 56 rotated 180°, shown in FIG. 7b, the upper leaf 56b no longer supports the

10   carrier and instead the carrier has dropped slightly and is supported by the lower leaf 56c. FIG. 8c shows the position of the cam 56 rotated 270° wherein the upper leaf 56b has rotated sufficiently to begin to engage the undercut 66 of the next slide carrier while the opposite

15   facing lower leaf 56c still supports the bottom carrier. After a full rotation of 360° as shown in FIG. 7d, the lower leaf 56c has rotated opposite the carrier stack and no longer supports the bottom carrier which now rests on the loading platform 52. At the same position, the upper

20   leaf 56b supports the next carrier for repeating the cycle.

Referring again to FIG. 6a & 6b, when the carrier drops to the loading platform 52, the contact closes switch 90 which activates motors 86 and 87. Motor 86

38

drives the infeed drive belt 59 until the pusher tab 58

makes contact with the carrier and pushes the carrier

onto the X-Y stage drive belt 50. The stage drive belt

50 advances the carrier until contact is made with switch

5   91, the closing of which begins the slide scanning

process described further herein. Upon completion of the

scanning process, the X-Y stage 38 moves to an unload

position and motors 8, and 88 are activated to transport

the carrier to the unloading platform 36 using stage

10   drive belt 50. Motor 88 drives outfeed gear 93 to engage

the carrier pitch rack 68 of the carrier 60 (FIG. 5b)

until switch 92 is contacted. Closing switch 92

activates motor 89 to rotate the unloading platform 36.

The unloading operation is shown in more detail in

15   end views of the output module 17 (FIG. 7a-7d). In FIG.

7a, the unloading platform 36 is shown in a horizontal

position supporting a slide carrier 60. The hold-down

mechanism 94 secures the carrier 60 at one end. FIG. 7b

shows the output module 17 after motor 89 has rotated the

20   unloading platform 36 to a vertical position, at which

point the spring loaded hold-down mechanism 94 releases

the slide carrier 60 into the output hopper 18. The

carrier 60 is supported in the output hopper 18 by means

of ears 64 (FIG. 5a and 5b). FIG. 7c shows the unloading

platform 16 being rotated back towards the horizontal

position. The platform 36 rotates upward and contacts

the deposited carrier 60. The upward movement pushes the

carrier toward the front of the output hopper 18. FIG.

5 7d shows the unloading platform 36 at its original

horizontal position after having output a series of slide

carriers 60 to the output hopper 18.

    The aspects of the apparatus 10 relating to scanning,

focusing and image processing are further described in

10 Patent Application 08/758,436 which is incorporated

herein.

Computer Implementation

    Aspects of the invention may be implemented in

15 hardware or software, or a combination of both. However,

preferably, the algorithms and processes of the invention

are implemented in one or more computer programs

executing on programmable computers each comprising at

least one processor, at least one data storage system

20 (including volatile and non-volatile memory and/or

storage elements), at least one input device, and at

least one output device. Program code is applied to input

data to perform the functions described herein and

generate output information. The output information is
applied to one or more output devices, in known fashion.

Each program may be implemented in any desired
computer language (including machine, assembly, high
5   level procedural, or object oriented programming
languages) to communicate with a computer system. In any
case, the language may be a compiled or interpreted
language.

Each such computer program is preferably stored on a
10   storage media or device (e.g., ROM, CD-ROM, tape, or
magnetic diskette) readable by a general or special
purpose programmable computer, for configuring and
operating the computer when the storage media or device
is read by the computer to perform the procedures
15   described herein. The inventive system may also be
considered to be implemented as a computer-readable
storage medium, configured with a computer program, where
the storage medium so configured causes a computer to
operate in a specific and predefined manner to perform
20   the functions described herein.

A number of embodiments of the present invention have
been described. Nevertheless, various modifications may
be made without departing from the spirit and scope of
the invention. Accordingly, the invention is not to be

limited by the specific illustrated embodiment, but only

by the scope of the appended claims.

CLAIMS

What is claimed:

1. A method for automated image analysis of a biological
specimen, comprising:

(a) providing a biological sample to be analyzed;

(b) automatically scanning the sample at a plurality
of coordinates;

(c) automatically obtaining an image at each of said
coordinates;

(d) automatically reconstructing an image of the
sample from each individual image to create a
reconstructed image of the sample; and

(e) processing the reconstructed image to identify a
candidate object or area of interest.

2. The method of claim 1, further comprising:

(a) automatically identifying a coordinate of the
candidate object or area of interest in the reconstructed
image; and

(b) automatically acquiring an image of the object or
area of interest, at the location coordinates obtained
from the reconstructed image.

43

3.   The method of claim 1, wherein the object or area of
interest is detected by immunohistochemistry.

4.   The method of claim 1, wherein the object or area of
5   interest is detected by in situ hybridization.

5.   The method of claim 1, wherein the object or area of
interest is detected by a stain.

10   6.   The method of claim 5, wherein the stain is a nucleic
acid dye selected from the group consisting of
hematoxylin, Giemsa stain, methyl green, Nuclear
Fast Red, Hoechst 33342, Hoechst 33258, thiazole orange,
DAPI, ethidium bromide, propidium iodide, TOTO, YOYO-1,
15   SYTOX Blue, SYTOX Green, 7-Aminoactinomycin, 9-Amino-6-
chloro-2-methoxyacridine, and acridine homodimer.

7.   The method of claim 5, wherein the object or area of
interest is stained with a cytoplasmic dye such as eosin
20   or Kleihauer_Betke cytochemical stain or a combination
thereof.

8.   The method of claim 1, wherein the object or area of
interest is a cell specific marker.

9.   The method of claim 8, wherein the cell specific
marker is detected by a nuclear stain and counterstain.

5   10. The method of claim 8, wherein the cell specific
marker is detected by immunohistochemistry, in situ
hybridization, staining or a combination thereof.

11. The method of claim 1, wherein the reconstructed
10 image is a digital image.

12. A method for histological reconstruction, comprising:
        (a) providing a sample of a biological specimen on a
series of slides, wherein consecutive samples are:
15   (1) stained by a nuclear stain such as
hematoxylin and eosin (H/E); or
                (2) detectably labeled by an
immunohistochemistry (IHC) method and counterstained; or
                (3) detectably labeled by an in situ
20 hybridization (ISH) method and counterstained; or
                (4) detectably labeled by a combined (IHC) and
(ISH) method and counterstained;
        (b) automatically obtaining , an image from each of
the samples

45

(c) automatically reconstructing the sample images such that a first sample image is paired with a consecutive sample having a corresponding image; and

(d) automatically performing a histological

5   reconstruction of the specimen.

13. The method of claim 12, wherein the first sample and consecutive sample have overlapping images.

10   14. A computer program, residing on a computer_readable medium, for automated image analysis of biological specimen, the computer program comprising instructions for causing a computer to:

a) process a sample, wherein the sample is stained

15   with a nucleic acid dye and a counterstain;

(b) scan the sample at a plurality of coordinates;

(c) obtain an image at each of said coordinates;

(d) reconstruct the sample from the individual images to create a reconstructed image of the sample;

20   (e) identify a coordinate of a candidate object or area of interest in the reconstructed sample; and

(f) acquire an image of the object of interest, at the location coordinates obtained from the reconstructed sample.

15. The computer program of claim 14, wherein the
reconstructed image of the sample is a digital image.

5   16. A computer program, residing on a computer_readable
medium, for histological reconstruction, the computer
program comprising instructions for causing a computer
to:

        (a) generate, digitize, and store in memory images
10 from a biological specimen on a series of slides, wherein
consecutive samples are:

            (1) stained by a nuclear stain such as
hematoxylin and with eosin; or

            (2) detectably labeled by an immunohistochemical
15 (IHC) method and counterstained; or

            (3) detectably labeled by an in situ
hybridization (ISH) method and counterstained; or

            (4) detectably labeled by a combined IHC and ISH
method and counterstained;

20   (b) place the stored sample images in an order such
that a nucleic acid stained and counterstained sample is
paired with a consecutive IHC or ISH sample; and

        (c) automatically perform a histological
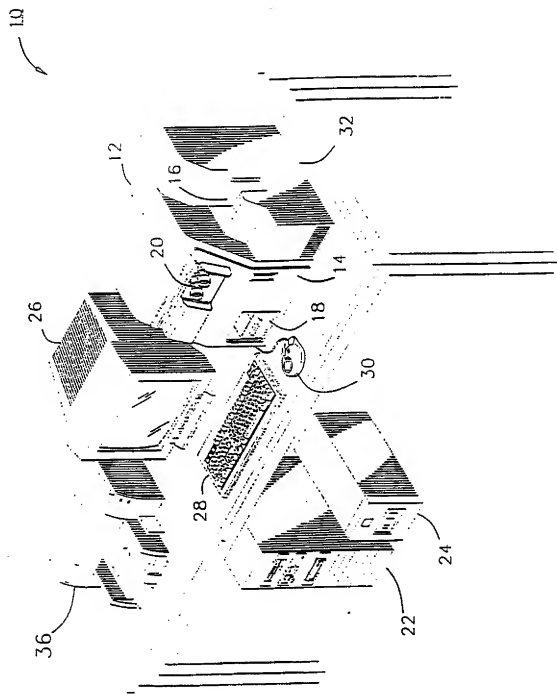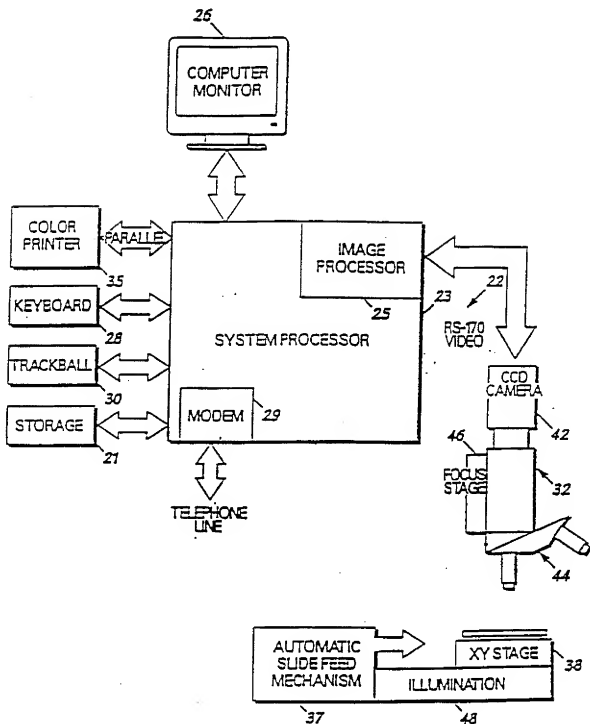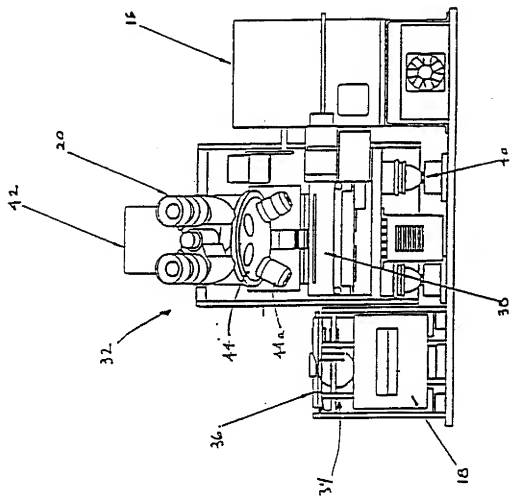reconstruction of the specimen.

fig 1

fig.2

Fig. 3
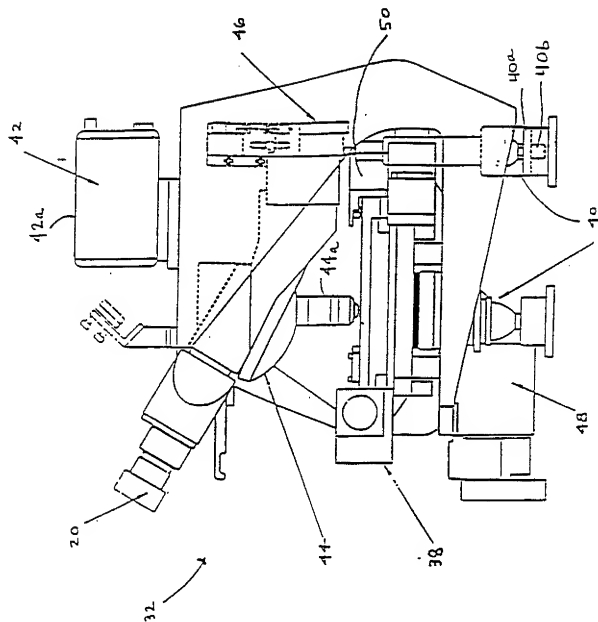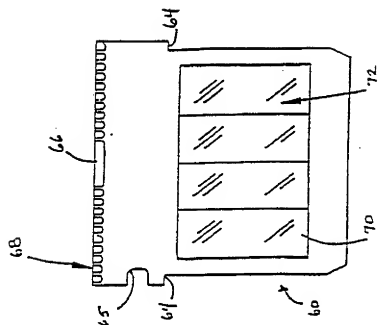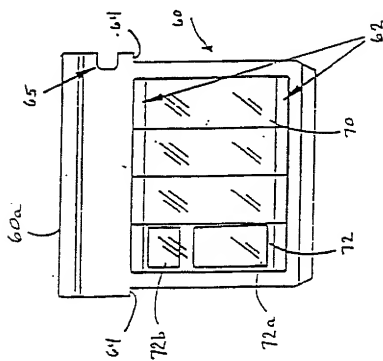
Fig. 4

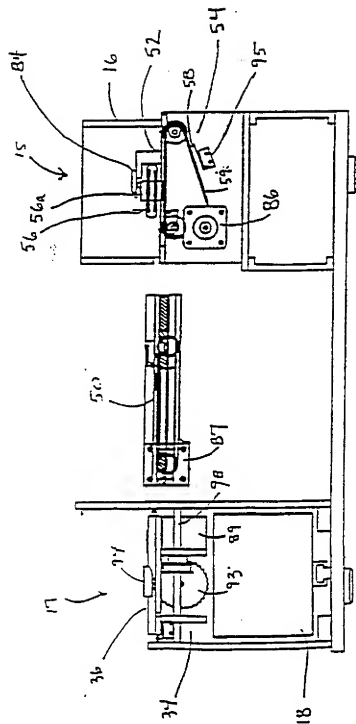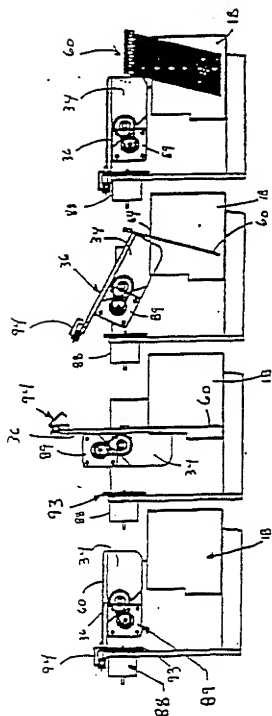Fig. 5b

Fig. 5a

FIG. 6a

FIG. 6b.

Fig. 7d

Fig. 7c

Fig. 7b

Fig. 7a

Figure 8: Find Specimen Algorithm

fig. 8

Fig. 9

301

Scanning Pattern

302

| Scan Field of View | Scan Field of View | Scan Field of View | Etc. |

302

Scanning Pattern

Etc.

Pattern of
building the
image

Direction reversed due to
optical flipping along the
vertical centerline

Etc.

Image of single field of view,
with a translation and scaling of
microns to pixels.

Etc.

Image of entire scan
area, with a translation
and scaling of microns
to pixels.
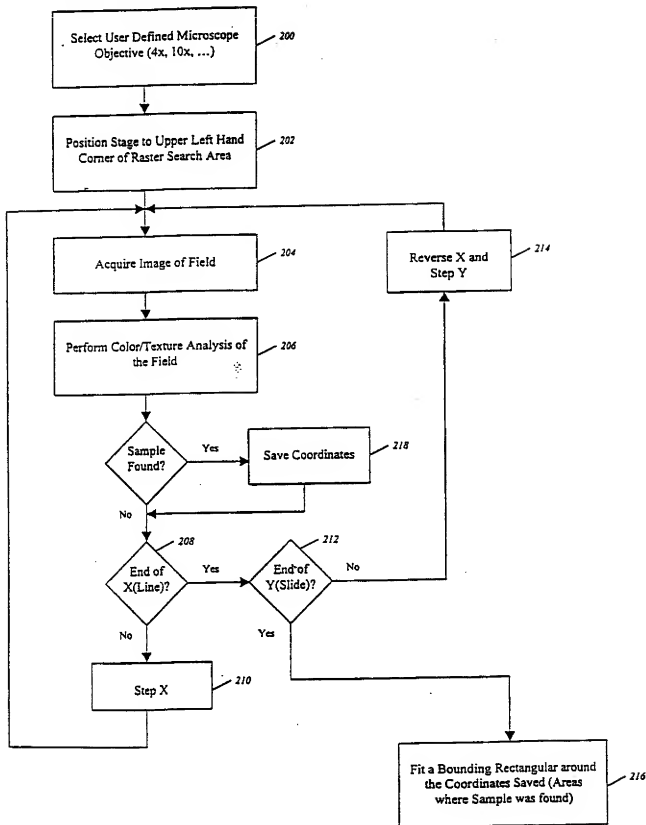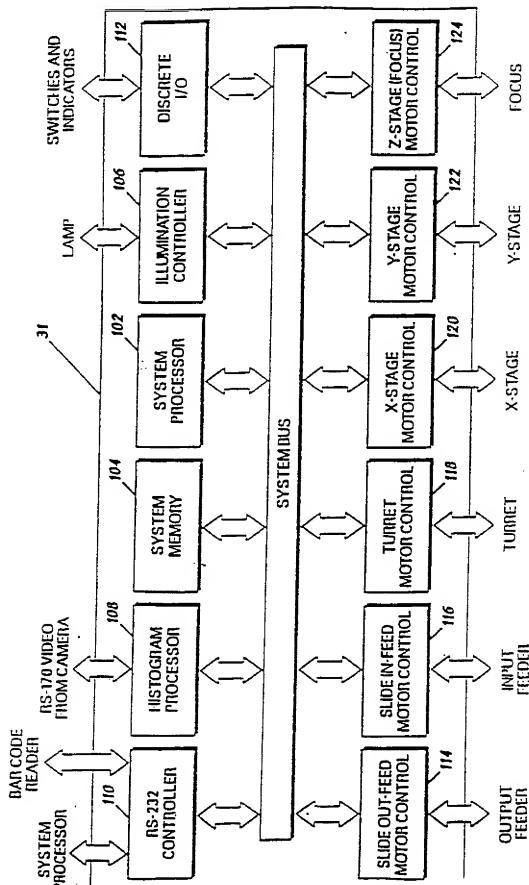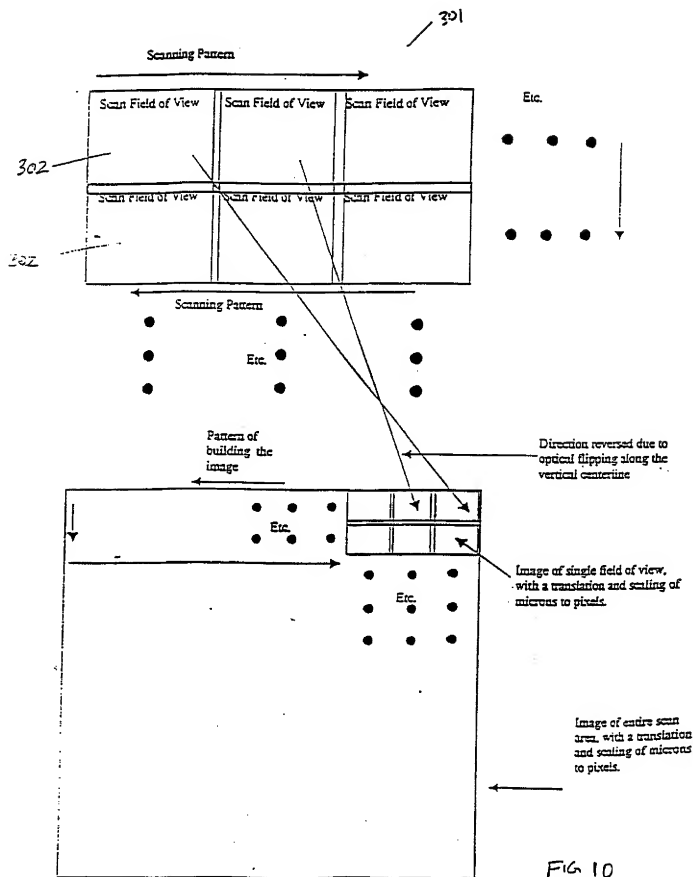
FIG 10

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : GO6K 9/56
US CL : 382/128-134, 308

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 382/128-134, 308

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

East

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,428,690 A (BACUS et al) 27 June 1995, col. 1, lines 12-49.	1-11, 14-15
X,P	US 6,007,996 A (McNamara et al) 28 December 1999, col. 1, lines 8-13; col. 34, lines 32-38.	3-4, 7-10,12-13,16

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 JUNE 2000	03 JUL 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KANJI PATEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0900

Form PCT/ISA/210 (second sheet) (July 1998) ★